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SHORT COMMUNICATION

Hepatitis C Virus Genomic Variability in Untreated and Immunosuppressed Patients

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To investigate whether immune pressure enhances the genetic diversity of the hepatitis C virus (HCV) hypervariable region 1, nucleotide sequences were compared in multiple sera, collected longitudinally, from three untreated patients and four patients undergoing liver transplantation for HCV-related cirrhosis. A minor variant became dominant in three of three patients following transplantation and persisted unchanged for months. Compared with untreated HCV carriers, transplant recipients had fewer quasispecies, fewer nucleotide changes (1.61 and 2.58/month), fewer amino acid sequence changes (0.40 and 1.94/month), as well as higher ratio of transitional to transversional mutations (2.57 and 0.98, $P < 0.02$) and lower replacement to silent mutations (1.33 and 8.21, $P < 0.01$). The two patients with the least genomic variation died of HCV graft infection. The data suggest that HCV variants which infect the graft are selected by recipient immune pressure at the time of transplant and that preferential replication in the graft is enhanced by routine immunosuppression.

Some individuals with chronic hepatitis C virus (HCV) infection develop cirrhosis and many will require liver transplantation (*1, 2*). Almost invariably, the liver allograft serves as target for HCV infection (*3, 4*). HCV is remarkable for its high degree of genomic variability in two discrete areas of the E2 structural region termed hypervariable region 1 and 2 (HVR1 and 2). Sequences from multiple cDNA clones of individual patient sera revealed a variable degree of genomic diversity within HVR1 and reflected in a population of viral mutants called quasispecies (*5–7*). To explore the role of immune pressure in the genomic variability of HCV, we compared the sequences of HVR1 in a group of patients who underwent liver transplantation for HCV-related end-stage cirrhosis and a reference group of patients with HCV-related chronic liver disease who did not receive any treatment.

Seven patients with HCV infection were included in the study (Table 1). All had antibody to HCV detected by screening with an enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL) and confirmed with RIBA2 (Ortho Diagnostics, Raritan, NJ). All were positive for HCV RNA detected by nested polymerase chain reaction (PCR) using previously described primer pairs in the highly conserved 5′ noncoding region (*8*). Patients 1–4 underwent one or two liver transplants during the course of the study subsequent to which they received standard immunosuppressive treatment with cyclosporine, prednisolone, and azathioprine. Patients 5–7 were apparently healthy blood donors with a past history of intravenous drug abuse.

RNA extraction was performed according to standard methods. Oligonucleotide primers for amplification of the HVR1 region and the 3′ end of the E1 region were used (*9, 10*): outer primers 3768 (5′ GGI CAY CGY ATG GCI TGG GA [sense]) and 3769 (3′ CIR TCG GTI GTI CCC ACB AC [antisense]) and the corresponding inner primers 3766 (5′ GGG AYA TGA TGA TRA ACT GG [sense]) and 3770 (3′ CAG TAI ACY GGR CCR CAY AC [antisense]). Type-specific sequence homologies contained within the amplified region enabled a simultaneous determination of the HCV type (*10*). Reverse transcription and first PCR were combined as described (*11*). The amplified PCR product was sequenced by the dideoxy nucleotide chain termination method (USB sequencing kit). Ten to 14 cDNA clones per patient sample were sequenced. The nucleotide sequence diversity in each set of sequences was calculated as the percentage of nucleotide differences from the dominant sequence of the initial sample. Replacement over silent (R/S) mutation ratios were calculated. In addition, amino acid sequences were deduced and conservative and nonconservative mutations were determined and analyzed.

Primers and probes for the quantitative nested PCR were selected in the core region. Outer primers were sense 5′-GCGCGAIAGGAAGACTTCG-3′ (481–499), antisense 5′-GATGTACCCCATGAGGTCGG-3′ (732–751); inner primers were sense 5′-CGTGGAAGGCGACAA-

Sequence data from this article have been deposited with the EMBL Data Library under Accession Nos. Z83699–Z83730.

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TABLE 1

Patient Characterization and Variability of HCV Nucleotide and Amino Acid Sequences

Patient	Age	Sex	Liver disease	HCV genotype	Samples studied					% nucleotide and (amino acid) variations from initial dominant variant sequences				
					1	2	3	4	5	1	2	3	4	5
1	56	M	CIR-HCC[a]	1b	−11[b] 2.5×10^4	8 5.0×10^5	86 7.0×10^6	218 5.5×10^6	329 7.0×10^6 [c]	2.1 (0.9)	6.1 (2.0)	3.4 (3.7)	5.2 (4.0)	5.0 (3.7)
2	66	M	CIR	1b	−33 5.0×10^6	88 7.0×10^8				1.8 (3.4)	7.1 (14.2)			
3	48	M	CIR	1b	31 1.0×10^5	97 5.2×10^6	180 ND	334 ND	465 ND[d]	2.7 (4.3)	2.3 (0)	0.3 (0)	0.1 (0)	1.4 (0.9)
4	46	F	CIR	1a	−33 $<1 \times 10^3$	47 4.5×10^5				0.4 (0.7)	3.7 (7.7)			
5	32	M	CAH	1a	0 ND	267 7.0×10^5				1.2 (2.1)	6.3 (12.1)			
6	44	M	CAH	2b	0 ND	457 8.0×10^6	719 6.0×10^6			4.9 (10.4)	16.4 (45.0)	12.9 (15.8)		
7	37	F	CPH	1b	0 7.0×10^6	24 1.5×10^7	87 5.0×10^6			2.1 (4.7)	15.4 (16.4)			

[a] CIR, cirrhosis; HCC, hepato cellular carcinoma; CAH, chronic active hepatitis; CPH, chronic persistent hepatitis.

[b] Time from OLT (− indicates prior to OLT) for patients 1–4 expressed in days; 0 corresponds to the first samples of patients 5–7 who did not undergo liver transplantation. For each sample the HCV RNA concentration is given in genome equivalent.

[c] HCV viremia expressed in genome equivalent per milliliter (see materials and methods).

[d] Not done.

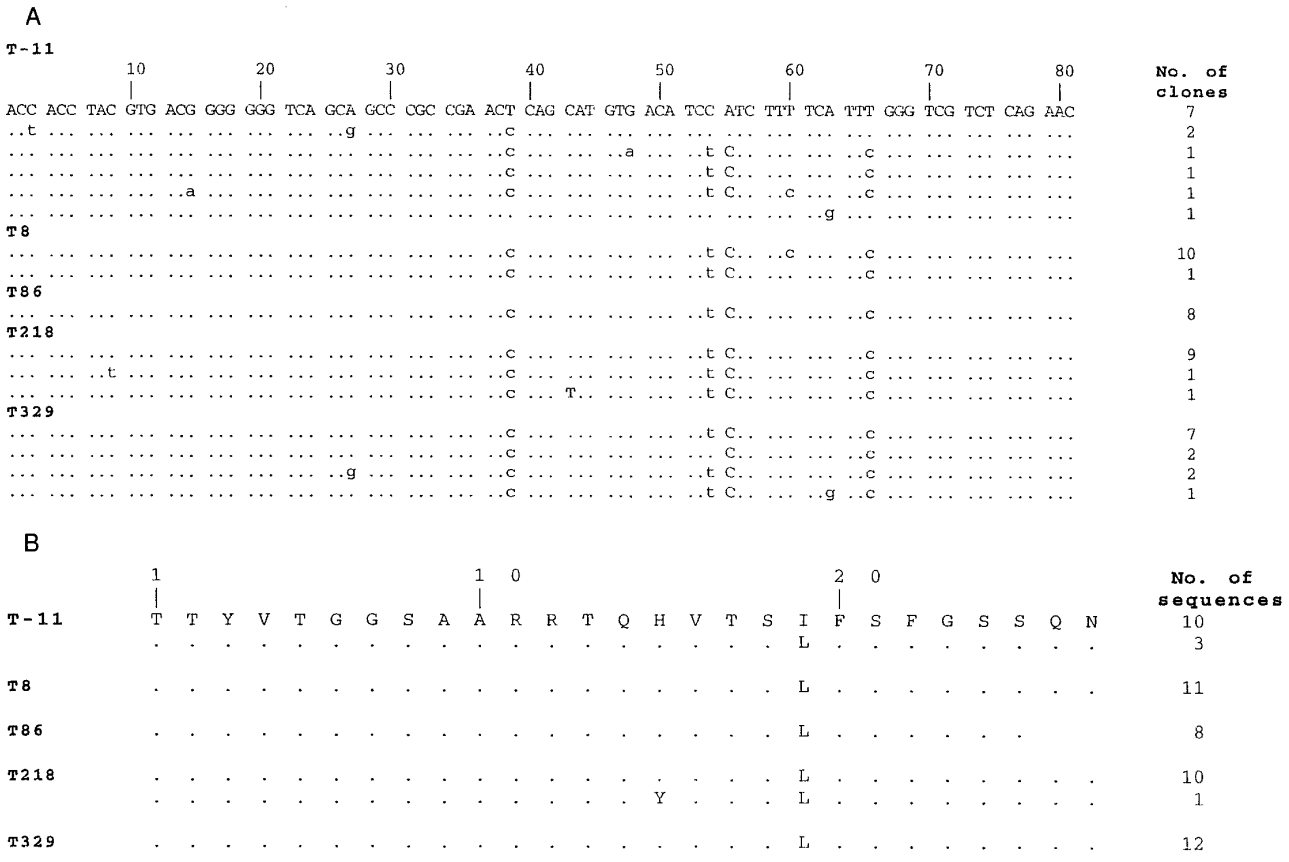

FIG. 1. (A) Nucleotide (nt) and (B) deduced amino acid sequences of the HCV HVR1 region of patient 1. Both sequences are aligned with the most frequently found variant (dominant quasispecies) in the first time point sample. Dots indicate identity with the nucleotide or amino acid lead sequence. Lowercase indicates silent and capital replacement mutations. Letter "T" followed by a number corresponds to time from transplantation in days. The number of clones (right-hand column) corresponds to the number of identical sequences found in each sample. "No. of sequences" indicates the number of identical deduced amino acid sequences in each sample. Nucleotide 1 corresponds to nt 1489 of HC-J8 (*9*).

CCTAT-3′ (515–535), anti-sense 5′-CGCATGTTAGGG-TATCGAT-3′ (706–725); probe was 5′-ATGGGCTGG-GCAGGATGGCTC-3′ (611–632), 5′ digoxigenin labeled. Quantitation was done by scanning bands after hybridization from a standard containing a known number of copies to draw a curve against which each sample was quantitated (*12*). When applicable, comparison of ratios was done using the Fisher exact test. A P value < 0.05 was considered significant.

As shown in Table 1, the levels of viremia pretransplantation and in untreated patients were similar. In contrast, approximately 3 months posttransplantation, levels of viremia increased 10 to over 100 times and tended to subsequently decline.

The HVR1 sequences of patients 1, 2, and 4 were determined within the month preceding and 2 to 3 months following transplantation (Fig. 1, Table 1). In each case, a minor variant or a closely related variant present prior to transplantation became dominant or exclusive following the procedure (Figs 1 and 2). In patient 1 only one of the five observed mutations led to a conservative amino acid replacement. In patients 1 and 4, mutations of pretransplant minor variants produced deduced amino acid replacements that persisted posttransplantation and were still detectable after 1 year of follow-up in patient 1 (Fig. 1). The HVR1 regions of patients 1 and 3 were sequenced in five serial serum samples over approximately 1 year (Figs. 1 and 2). In both cases, only silent mutations were observed in the dominant variant, and the deduced amino acid sequences remained constant. This relative lack of mutation contrasted markedly with results from patients 5 and 6 who had not received immunosuppressive treatment and were followed-up over a similar time interval. In both cases, mutations corresponded to the occurrence of nine deduced amino acid replacements of the 27 total amino acids of the HVR1 region (Table 1).

The HVR1 nucleotide and amino acid sequence diversity in four immunosuppressed and three untreated patients was compared. As shown in Table 1, the average number of variants per sample was lower in the immunosuppressed patients (range 1–6, mean 3.14) than in the nontreated patients (range 2–10, mean 4.5). This difference was even more marked when samples preceding immunosuppression were discounted (mean 3.0). Taking as reference the dominant variant sequence of each initial sample, the mean percentage of nucleotide variation was 3.5% for the 10 follow-up samples of immunosup-

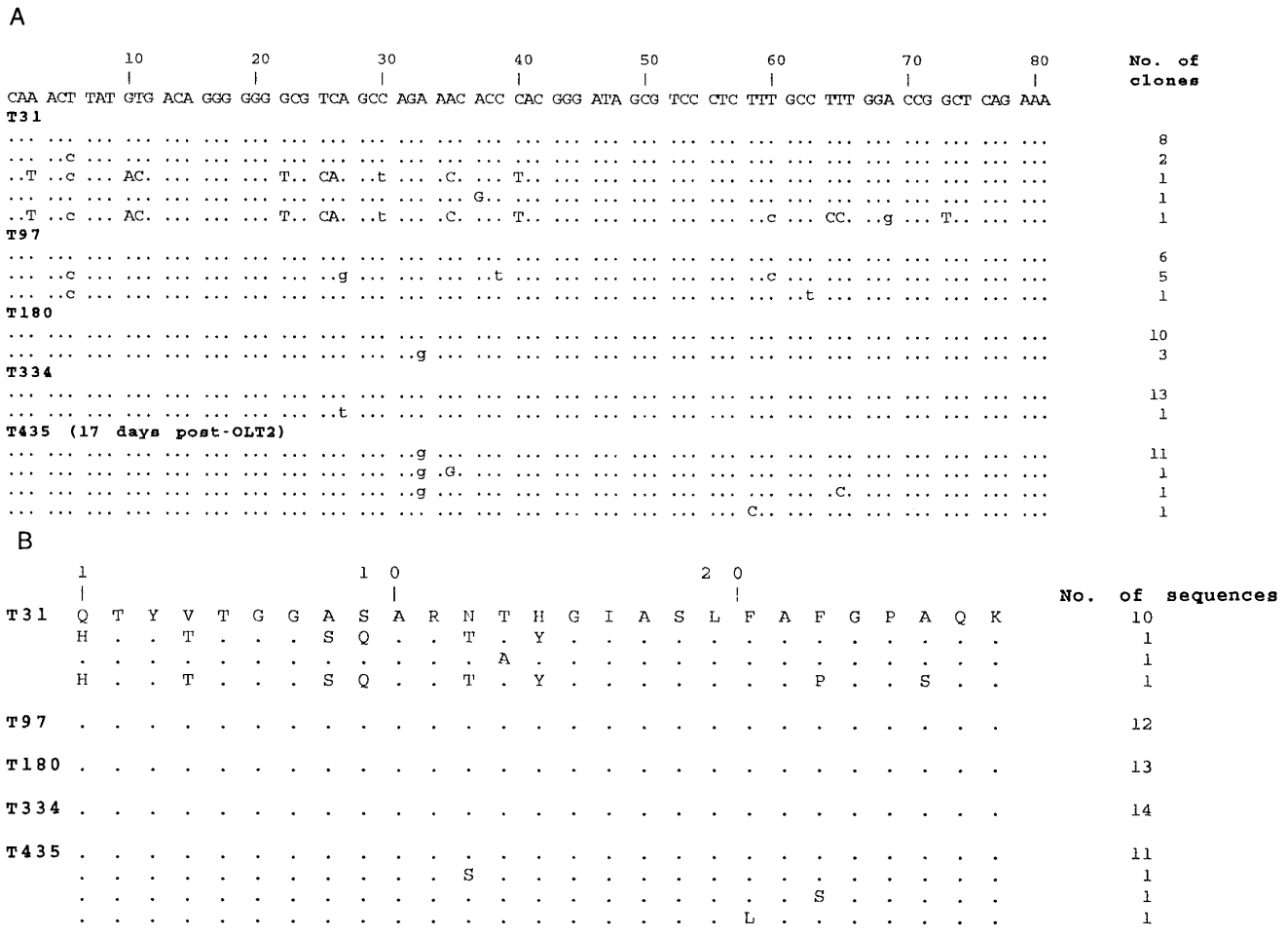

FIG. 2. (A) Nucleotide and (B) deduced amino acid sequences of the HCV HVR1 region of patient 3. See legend to Fig. 1.

pressed patients and 13.5% for the 5 samples from the untreated patients. When the periods of observation were summed up for each group of patients, untreated patients and liver transplant recipients had averages of 2.58 and 1.61 nucleotide substitutions per month, respectively. The ratio between transition and transversion of nucleotides was significantly different: 0.98 and 2.57 ($P < 0.02$) in the untreated and immunosuppressed groups, respectively. The ratio R/S was significantly higher in untreated patients (8.21 vs 1.33, $P < 0.01$).

A total of 259 HVR1 sequences were obtained, 85 from untreated patients and 174 from transplanted patients. The respective positions of these mutations for each patient in these two groups were plotted. Of the 81 nucleotide positions of the HVR1 sequence, 24 (30%) were conserved, 12 of them encoding five conserved deduced amino acids at positions 2, 6, 7, 23, and 26. All other areas had a degree of variability. This was relatively high in two areas (hot spots) for both groups of patients: nucleotide positions 25–40 and 62–65. In contrast, the region spanning nucleotide positions 43–55 was largely variable in the untreated patients but not in the immunosuppressed patients.

Both patients 1 and 3 required a second liver transplantation, 14 and 13 months after the first transplantation, respectively. In contrast, patients 2 and 4 had uncomplicated transplantation outcome after 20 months and 1 year of follow-up, respectively.

The main feature of the comparison of pre- and post-transplantation distribution of quasispecies is the apparent selection of a minor quasispecies to infect the transplanted liver (Fig. 1). This phenomenon can be explained if the minor quasispecies are more infectious escape mutants unrecognized by the patient immune system at the time of transplantation (*13*). In patients 1, 3, 5, and 6, two samples collected at approximately 1-year interval were examined. In the two immunosuppressed patients (Figs. 1 and 2) there were 0 and 3 mutated nucleotide positions, while in the two patients who followed a natural history course, 14 and 23 were observed. This was consistent with results of other groups (*14*). In untreated patients 5 and 6, 10 and 15 amino acid replacements were observed, respectively. These numbers were markedly higher than 0 and 3 substitutions observed in patients 1 and 3. These results are consistent with previous reports (*6, 15, 16*). Most frequent nucleotide substitutions were limited to two hot spots of the HVR1: nucleotides 25 to 40 and 62 to 65, but were extended to nucleotides

25–66 in both patient groups. Except for the nucleotides 25–27 coding for amino acid 9, the mutation rate in untreated patients and to a lesser extent in immunosuppressed patients is the highest at positions coding for the HVR1 epitope I described by Kato *et al.* (*17*) (nucleotide 31–63). The highly variable nucleotides at positions 64–66 code for the first of 3 nonoverlapping amino acids of epitope II. Upstream and downstream nucleotides are overall more conserved in both patient groups. It has been argued that the relative infidelity of Amplitaq may influence sequencing results and some investigators used more than one amplicon. We considered that the degree of error introduced by Amplitaq was insignificant relative to the frequency of mutations observed.

As shown in Table 1, all criteria examined to compare HVR1 sequences revealed a difference between immunosuppressed and untreated patients. The average number of quasispecies, mutation rate, and replacement/silent mutation ratio were lower in the immunosuppressed group. In addition, mutations tended to be mostly transitional and more often silent, while replacement mutations tended to favor conservative substitutions. All these elements suggest that a normal immune system plays a critical role in the determination of the quasispecies distribution of HCV-infected individuals. It is unlikely, however, that immunosuppression alone accounts for this remarkable difference. One possible additional influence is the individual patient's immune repertoire. Patients who reach the stage of terminal liver disease at a relatively young age may represent a selection of people whose immune response, both humoral and cellular, was particularly inefficient (*18, 19*).

The observed low HVR1 genomic variability may have preexisted the period of immunosuppression and be related to the end-stage liver disease requiring liver transplantation. It is possible that the reduced viral diversity observed in cirrhotic patients who underwent liver transplantation is due to a host inability to contain HCV, hence the cirrhosis, or to the immunosuppressive treatment given for transplantation or both. This last hypothesis may be correct as some differences in replacement/silent mutation ratios and frequency of amino acid substitutions between the two patient groups were already present prior to immunosuppression. After a few months of immunosuppression, these differences were enhanced.

Previous reports have found high levels of viremia posttransplantation (*3, 4*) and we and others (*5*) observed a restricted number of quasispecies. At the time the new liver is transplanted, a replicative advantage is offered to a minor quasispecies (escape mutant) which massively infect the new, infection-free, target organ (*4*).

In conclusion, we present evidence for different patterns of HCV genomic drift in immunosuppressed and untreated patients with chronic liver disease. They strongly suggest that temporal changes in viral species distribution is largely driven by immune pressure since the removal of this pressure limits the viral diversity.

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